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STRUCTURAL AND FUNCTIONAL PROPERTIES AND BIOLOGICAL ACTIVITY OF THE CAPSULE ANTIGEN, "MURINE" TOXIN AND YERSINIA PESTIS ENDOTOXIN

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STRUCTURAL AND FUNCTIONAL PROPERTIES AND BIOLOGICAL ACTIVITY OF THE CAPSULE ANTIGEN, "MURINE" TOXIN AND YERSINIA PESTIS ENDOTOXIN

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The capsule antigen, the "murine" toxin and endotoxin /91** are important structural components of the Y. pestis cells. The participation of these antigens in implementation of plague pathogen pathogenicity is generally acknowledged. Since they were produced in relatively pure form a large /92 number of often contradictory works have been published studying the physical and chemical properties, biological, serological, protective activity and genetics of these substances. The volume of this article does not allow us to encompass all of these aspects. We focused primary attention on analyzing the data that have been accumulated by now regarding the structure and biological properties of these antigens.

Capsule Antigen

During cultivation both in vivo and in vitro at 37°C, the Y. pestis cells produce a capsule substance, the primary component of their surface structure. Attempts to isolate this substance in the pure form have been underway since the 1940's. Using settling of an aqueous extract of plague microbe cells by ammonium sulfate in the 20 - 40% saturation zone, Baker, et al. [17] produced a protein-carbohydrate complex and protein that they designated FIA and FIB. T. T. Cocker, et al. [24] noted the good solubility of the capsule antigen

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in water. In studies using scanning electron microscopy, T. H. Chen and S. S. Elberg [23] demonstrated that the capsule antigen on the surface of the bacteria forms a granulation layer which gradually diffuses into the environment. The joint use of the immunoferritin method and electron microscopy allowed K. G. Zolotarev, et al. [10] to confirm these data and to demonstrate localization of FI not only on the surface of the cellular wall, but also on the membrane. The authors advanced the hypothesis that FI components are synthesized on the membrane-ribosomal complex and are finally accumulated on the cytoplasmic membrane of the microbe cell.

In order to isolate the capsule antigen, in addition to the traditional method of settling by ammonium sulfate we also used such sparing methods of producing protein as electrophoresis in gel, ion-exchange chromatography and gel-filtering. In this case W. Habig, et al. [33] obtained a fraction of FI consisting of a heterogenic population of proteins formed by a series of macromolecular units. For study in the polyacrylamide gel in the system of sodium dodecylsulfate, the units are fractionated into simple antigen subunits containing proteins and glycoproteins with molecular weight ranging from 15,000 to 17,000 [18]. These data were confirmed by the research of L. N. Serdobintsev, et al. [13] who established that the macromolecule of capsule antigen (which is essentially a pure protein) at the level of the quaternary structure is formed by subunits with molecular weight of 13,500. The dissociated subunits could again cluster into complexes of varying molecular weight. The authors draw an analogy between FI and the surface structures of different microbiological objects such as the protein membrane of the virion capsid or flagella of E. coli. At the same time R. Glosnika and E. Gruszkiewicz [31] provided proof for a

qualitatively different structure of the FI component that is responsible for its antigen and biological activity. Using enzymatic digestion by pancreatin, column chromatography containing a mixture of membranes of human erythrocytes with celite, and rechromatography on sephadex G-200, the authors produced a homogeneous preparation of capsule antigen that has high specificity in serological reactions and receptor activity in relation to the plague microbe phage. Study of chemical and physical properties of the antigen demonstrated its lipopolysaccharide nature. It was demonstrated that the carbohydrate part of the preparation consists of galactose and fucose. The lipid fraction contains phosphatidilethanolamine and phosphatidilserin. According to preliminary data, lipopolysaccharide (LPS) FI differs from FI of the Y. pestis wall because it lacks glucose, hexo samines and has ethanol amine. In order to stress the differences in the LPS FI and endotoxin of the plague pathogen, the authors propose designating it by the term "galactolipid." The opinion is advanced that the polysaccharide portion is responsible for antigen specificity of galactolipid. From a preparation of capsule antigen obtained by settling by ammonium sulfate [14] by the method of Westphal-Luderitz we isolated an LPS that has significant viscosity and does not cause lethal shock in mice during intra-abdominal /93 injection in doses of 1 - 2 mg.

The capacity of the capsule substance of many types of microorganisms to prevent attraction and absorption of them by professional phagocytes is generally known. The capsule substance usually has less pronounced antigen properties than the cellular components proper, therefore it masks the antigens of the cellular surface, inhibiting their bonding with IgG and Fc-receptors of the phagocytizing cells [35]. In addition, the capsules of the gram-positive microorganisms

have hydrophilic properties that impair their phagocytosis [44]. It is customarily believed that the antigen of Y. pestis membrane also is capable of preventing absorption of bacteria by phagocytizing cells. Consequently, the presence of FI is viewed as one of the important indicators for virulence of the plague pathogen. The results of studies, however, do not allow us to unequivocally support this hypothesis. There are publications which indicate that neutrophils and macrophages of animals sensitive to plague absorb cells FRA^- more intensively than cells FRA^+ of the plague microbe strains [15, 47]. FI preparations suppress the absorbing activity of phagocytes [9, 46]. On the other hand, no correlation has been noted between the degree of absorption of Y. pestis by neutrophils and macrophages in vivo and in vitro and the presence or absence of FI [21, 27]. A number of studies conducted in the 1950's [20] and later [12] did not note the capacity of FI to suppress the absorbing activity of phagocytes. There are data that there is no relationship between the virulence of the plague microbe strains and the presence in them of FI [2].

Pathogenic microorganisms are separated into two groups according to the site of their survival. The first have the capacity to survive and reproduce outside the phagocytes or cells of other types. The second which include the plague pathogen reproduce only within the cells. It is obvious from here that attraction and absorption of the Y. pestis bacteria by the phagocytizing cells are necessary to preserve their viability. Successful transformation of these functions by the phagocytes is guaranteed by the presence on their surface of specialized structures, as well as definite environmental conditions. It is natural to hypothesize that the cells of the plague microbe have the capacity to activate the initial phases of the phagocytic reaction. In our opinion, the

material carrier of this capacity could be the membrane substance of the plague pathogen. From this viewpoint the biological meaning of the presence of LPS on the surface of the plague microbe and its diffusion into the environment becomes understandable. Activation of the complement system by alternative path is thus viewed as one of the main properties of the LPS. In this case there is formation of components of the complement characterized by pronounced hemotaxic and opsonic activity [30]. In addition, according to the data of M. B. Goren [32], the lipid-containing structures that are on the surface of the microbe cells give the latter pronounced hydrophobic properties, and at the same time facilitating their contact with the surface of the phagocyte and subsequent absorption.

"Murine" Toxin

Cells of the plague microbe produce a toxic protein that is lethal for mice and rats and is known as "murine" toxin, or FII [17]. E. Englesberg and G. Levi [28] were some of the first to produce a purified toxin preparation. At the early stages of study FII was classified as exotoxins, however subsequently the link between "murine" toxin and the membrane of the bacterial cell and the possibility of its entering the environment only as a result of bacterial autolysis [41] were demonstrated. Under optimal conditions of cultivation, the FII content reaches 7% of the dry microbe mass [16]. By using settling by ammonium sulfate in the 30 - 70% saturation zone with subsequent fractionation, T. C. Montie and D. C. Montie [42] obtained from the extract of plague microbe cells a highly purified preparation of "murine" toxin consisting of two protein fractions: A with molecular weight 240,000 (10.9 S) and B 120,000 (7.6 S). Both FII proteins are characterized

by high content of acid amino acids. About 50% of the amino acid residues of the toxin fractions are hydrophobic amino acids. Under the influence of sodium dodecylsulfate, polymer proteins A and B dissociate into five or ten subunits with molecular weight 24,000 with one disulfide bond. Each /94 subunit consists of protein chains with molecular weight 12,000. It is important to note that the FII subunits with molecular weight up to 12,000 maintain biological activity inherent to the polymer molecule. The authors assume that dissociation of the units of "murine" toxin could occur on the surface of the bacterial cell.

Compared to protein A, fraction B contains 33% less tryptophan. The authors link precisely this difference to the greater toxic activity of protein A. Introduction into the medium of substances bonding SH-group (Ag^+, Hg^{2+}), or analogs of tryptophan, for example 4-methyltryptophan severely reduces the toxic activity of FII. Based on this and some other proofs, the authors believe that the toxophoric center of the biologically active oligomer is located in the site near the SH-groups and includes at least one residue of tryptophan. Indicating the differences in properties of proteins A and B, we should note many common features. For example, both proteins are thermally labile, resistant to the effect of trypsin and do not lose activity in a 0.05 - 0.1% solution of sodium dodecylsulphate. Their properties are fairly identical to hypothesize the origin of both fractions of toxin from the general structure of the cytoplasmic membrane of the plague microbe.

LD_{50} with parenteral injection of a purified preparation for rats is 0.08 mg per 1 kg of weight [36], and for mice varies from 0.7 to 6 μg/kg with intra-abdominal injection and

from 0.2 to 2 μg/kg with intravenous injection [8, 17]. The impact of purified toxin preparations on the mitochondrial respiration of heart and liver cells was studied in the series of experiments conducted in 1958-1966. The results summarized in the survey of S. Kadis and S. J. Ajl [37] allow us to assert that the toxin is capable of inhibiting respiratory activity of the mitochondria, reducing activity of the enzyme $NADP_2$-coenzyme reductase, at the same time blocking the system of electron transport. The toxin causes swelling of the mitochondria and change in their capacity for accumulation of the calcium ions and inorganic phosphate. It has been demonstrated that mitochondria of the heart and liver cells of rats are sensitive to the effect of the toxin, while the toxin does not have an impact on the mitochondria of rabbit heart cells. Based on the change in the EKG pattern during parenteral injection of "murine" toxin to rats, the authors hypothesized that cardiac insufficiency caused by blockage of mitochondrial respiration was the cause of death of the animals. Data obtained in vitro whereblockades of respiratory activity of a mitochondrial suspension require high concentrations of toxin, from 0.5 to 2 μg/ml compared to 0.5 - 3 μg of preparation leading to the death of mice, however, are discouraging. G. L. Hildebrand, et al. [36] believe that cardiac insufficiency is not a direct cause of death of the sensitive animals who received FII. Studies in this trend therefore did not allow us to establish the cause for the fatal outcome of "murine" toxin.

Experiments conducted in the second half of the 1970's in the laboratory of C. Montie (Department of Microbiology, University of Tennessee, Knoxville, USA) led to data necessary for an understanding of the operating mechanism of "murine" toxin. The researchers link the damaging effect of toxin to

its capacity for direct metabolic blockade of β -adrenergic receptors by means of interaction between the SH-groups and the residues of tryptophan. It is likely that toxin is capable of destroying the disulfide bonds of the receptor [42]. The "murine" toxin blocks such aspects of epinephrin activity as the capacity for endogenous and exogenous mobilization of free fatty acids in blood and the capacity to cause hyperglycemia. In this case one of the main results of research was establishment of the reverse relationship between the activity of toxins and the level of cyclic adenosine monophosphate (cAMP). All physical and chemical factors causing decrease in toxin activity, at the same time causing increase in the level of cAMP, and on the contrary, decrease in the level of cAMP correlate with increase in the toxic /95
effect. Increasing the temperature of maintaining the animals to 37°C, parenteral injection to the mice of cortisone, glucagon and sublethal doses of cholera toxin that are activators of adenylate cyclase system thus lead to higher resistance to the lethal effect of "murine" toxin. Glucagon and cAMP are capable of removing the toxic blockade of β-adrenergic system. The terminal consequence of the blockade is the inability of the toxin-sensitive animals to generate an adequate quantity of heat according to the ambient temperature. Inhibition of thermogenesis and overall loss of heat by the body lead to hypothermia and death of mice on the background of lower level of free lipid acids in the plasma. Introduction of "murine" toxin also leads to vasodilation of the peripheral vessels, which together with hypothermia also fosters a lethal outcome [19, 40, 51].

The effect of "murine" toxin has a great similarity to synthetic blockers of the β-adrenergic system such as dichloroisoproterenol and Propranolol [40]. In the operating features

of other blockers we can also find a similarity to the impact of FII. For example, dibencyline inhibits the activity of epinephrine in rats [45], but not in dogs [38]. T. C. Montie [40] believes that the species specificity of the "murine" toxin completely determines its action as an adrenergic antagonist.

The data on the impact of FII on other systems of the body of mammals are few and contradictory. The value of the "murine" toxin in plague pathogenesis also remains obscure. In addition to the statement of the "extremely active role in plague" [8] in the literature there are indications that toxin formation does not have any relationship to virulence of the plague microbe [2], and that FII is not the leading factor in plague pathogenesis, even in sensitive animals [5].

Endotoxins

Endotoxins are a large class of macromolecules that are representatives of LPS and are closely linked to the outer membrane of gram-negative bacteria. Despite the diverse composition, the LPS of different microorganisms have a similar structure. In the LPS molecule there is a hydrophobic lipid area, lipid A and covalently-bonded hydrophilic heteropolysaccharide part which is usually branched in which the core and the O-specific polysaccharide chain are distinguished [52].

In 1956, D. A. L. Davies [25] by using the method of water-phenol extraction for the first time obtained a specific lipopolysaccharide complex of Y. pestis containing lipid A and heptose. A more detailed study of the chemical structure and physical properties of LPS of the plague microbe was conducted by J. L. Hartley, et al. [34]. According to the data

obtained by these researchers, the molecular weight of LPS <u>Y. pestis</u> is 1.6 x 10.8. A total of 29.2% of the overall mass of the endotoxin is lipid A. The lipid includes glucosamine and glucosamine-6-phosphate. It has traces of ethanol amine and only one lipid acid, hydroxymiristat that is connected to glucosamine by an amide bond. The structure of lipid A is generally more complicated than similar structures of gram-negative bacteria. The total quantity of carbohydrates is 52% of the total mass of the LPS molecule. Carbohydrates of the core are represented by D-glucose, L-glycero-D-mannoheptose, D-glycero-D-mannoheptose, glucosamine and 2-keto-3-deoxy-D-mannooctonate. These carbohydrates, with the exception of D-glycero-D-mannoheptose are included in the core of the microbes that belong to the family Enterobacteriaceae. This latter carbohydrate is not unique; it is found in the core of endotoxins of at least seven types of gram-negative bacteria. As the authors believe, the polysaccharide part of the molecule consists of residues of neutral sugars interconnected by several types of glycoside bonds, mainly in the β-configuration. Pronounced branching is inherent to the structure of the polysaccharide.

It is hypothesized that the plague microbe has a defect in synthesis of full-fledged LPS characteristic for the S-forms of gram-negative bacteria, and for this sign the LPS of its wall is closer to the R forms which have an insufficiently built structure of endotoxin [3]. The opinion has been advanced that cells of the plague pathogen, maintaining capacity to /96
synthesize the O-specific chain of polysaccharide cannot add it to the molecule core [4]. Processing of the endotoxin preparation with sodium dodecylsulfate reduces the size of the molecule all the way to insoluble fragments [34] which confirms the opinion of D. A. L. Davies [26] about the highly

unitized nature of the endotoxin.

Immediately after obtaining the LPS Y. pestis its significantly lower capacity to cause lethal shock in the animals compared to endotoxins of other gram-negative bacteria [25] was demonstrated. Data about the relatively weak toxicity of the plague pathogen LPS was subsequently repeatedly confirmed. The researchers obtained LD_{50} values of the endotoxin for albino mice from 750 [22] to 3000 g [50]. According to the data of V. P. Avrorov and A. V. Griboyedov [1], guinea pigs, then white rats and white mice are the most sensitive to the plague microbe endotoxin, while small ground squirrels are the most resistant.

The LPS of the plague pathogen, like the endotoxins of other microorganisms is a polyfunctional preparation that causes diverse changes affecting many systems in the body. Preparations of the LPS Y. pestis thus induce a fairly significant inflammatory skin reaction in intact animals [6]. In the regional lymph nodes in white mice within 24 h after injection of LPS, hyperplasia, necrosis of the tissue components and infiltration by leukocytes are observed. By this time in the spleen there is hyperemia, symptom of stasis in the sinuses of the pulp and necrosis of the lymph elements are noted. A very characteristic feature of the effect of endotoxins is early affection of the blood vessels [11], increase in capillary brittleness, thrombosis and intravascular coagulation [29], collapse and hemoglobin-impregnation of the vascular walls [48]. Brain tissue and cardiac muscle are often involved in the process in mice and guinea pigs. Hemorrhagic affections of the subcutaneous cellulose, intestinal tract, peritoneal wall and adrenal glands are noted. Acute necrosis of the real glomeruli and vacuolization of the parenchymal

cells of the liver is similar to acute fatty degeneration [11, 48, 50] are observed.

After injection of the endotoxin, lower content of acid and alkali phosphatases (within 12 - 24 h) occurs very early in the experimental animals. In the case of the animal's death, the phosphatase content is diminished so much that they are only found in traces in the body. The quantity of glycogen in cells of the liver and glucose in the blood is severely reduced. A decrease was established in the content of nucleic acids, and the content of RNA changes most noticeably. An increase is indicated in the content of arginine and nitrogen in the blood [11, 48]. Injection of endotoxin induces temperature changes in the body [25, 48] and leads to a decrease in the number of neutrophils in the blood with subsequent neutrophilia [7]. It is appropriate here to stress that injection of the LPS of the plague pathogen in overall features reproduces the pattern recorded during plague infection [11, 49].

Some researchers believe that the primary targets for the effect of the plague microbe endotoxin are polymorphonuclear glucocytes and cells of the monocytic phagocytizing system [7, 39]. This opinion corresponds to the currently accepted nypothesis according to which the target cells for endotoxin are macrophages stimulated in the presence of LPS to synthesize and release prostaglandins that cause such reactions typical for the endotoxin as fever, early hypertension and fatal shock [43].

With regard for the already obtained results, we should note the relative degree of limitation of the data on the biological effect of endotoxin of Y. pestis. Until now such important aspects of its action as impact on the system

of the complement, factors of the blood coagulation system, function of the phagocytizing cells, mitogenic activity of the LPS Y. pestis, capacity to induce synthesis of prostaglandins, induction of interferon production and so forth have not been studied. However even at this stage of research the endotoxin is considered one of the most biologically active substances of the plague microbe which has the leading role in plague pathogenesis [29, 49].

We should note in conclusion that the experimental data /97 obtained in recent years in the study of toxic substances of microorganisms, as well as development of basically new research create prerequisites for comprehensive study of the structure, biological activity and participation in the pathogenesis of plague of the primary antigens of Y. pestis which include capsule antigen, "murine" toxin and lipopolysaccharide complex.

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